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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Title

Isolation and culturing of fetal cells.

Introduction

The present invention relates to a method for isolating fetal cells from maternal blood, in particular from maternal blood not having been subjected to concentration or enrichment.

In addition the invention relates to a method for culturing fetal cells.

Background

The examination of fetal cells for early detection of fetal diseases and genetic abnormalities is carried out in connection with many pregnancies, in particular when the maternal age is high (over 35 years) or where genetic diseases are known in the family. Fetal cells may be obtained by amniocentesis, the removal of amniotic fluid from the amniotic cavity within the amniotic sac or by chorion biopsy, where biopsies are taken from the placenta.

During pregnancy a variety of cell types of fetal origin cross the placenta and circulate within maternal peripheral blood. The feasability of using fetal cells in the maternal circulation for diagnostic purposes, has been hindered by the fact that fetal cells are present in maternal blood in only very limited numbers, reported numbers have been from $1:10^6$ to $1:10^8$. In addition most fetal cells cannot be distinguished from maternal cells on the basis of morphology alone, but rather must be identified based upon detection of fetal cell markers. However, it would be advantageously to perform fetal diagnostics by a less invasive procedure, such as a maternal blood sample.

One particular fetal cell type within maternal blood that has been demonstrated to be useful for detecting fetal DNA is the nucleated erythrocyte.

Also, fetal granulocytes have been reported to be present in maternal blood. Granulocytes are one subpopulation of white blood cells found in the blood. There are three types of subsets of granulocytes (which also are referred to as polymorphnuclear leukocytes): neutrophils, basophils and eosinophils. All granulocytes have a distinctive morphology characterized by the nucleus and cellular granules.

Due to the very limited number of fetal cells in maternal blood concentration or enrichment of the maternal blood sample with respect to the fetal cells have been conducted by negative selection, i.e. removal of maternal cells. Enrichment of fetal cells by density gradient centrifugation or by removing maternal cells with an antibody to a cell surface antigen is described in for example US 5,858,649, US 5,731,156, US 5,766,843 and US 5,861,253.

Yet another method of removing maternal cells, in particular maternal erythrocytes, is by lysing, again optionally combined with immunologic methods for removing the maternal cells.

Another selection procedure is positive selection, for example by use of CD71 antibodies.

US 5,861,253 describes enrichment either before and/or after labelling of the fetal cells for further analysis.

It is however, a problem that due to the enrichment procedures some of the fetal cells may also be removed leading to even fewer fetal cells in the blood sample to be analysed.

In order to increase the number of fetal cells attempts of culturing the cells have been carried out in the prior art. There are a few publications describing successful methods for culturing fetal cells from peripheral blood of pregnant women. Lo et al, (Lancet 1994, 344, 264) cultured cells from five pregnant women carrying male fetuses. In two cases they examined samples before culturing with negative results. In all cases they ascertained cells by Fluorescence in situ hybridization (FISH) after culture. Very few details of the culture methodology are given, and the results have not been repeatable with the available information. In a study by Little et al, (Blood,

273, R 1829) culture was used as part of their isolation method and male cells were found aft r various sorting/enrichment procedures. Also Jansen et al, (Diagn., 1999, 19, 323) developed a method for culturing cord blood cells in a model system for isolation of fetal nucleated red blood cells. However, none of the methods have shown a significant increase in fetal cells after culture.

Summary of the invention

It is an object of the present invention to provide for a method for isolating fetal cells from maternal blood, wherein the blood sample has not been substantially enriched, such as by performing, on a sample of maternal blood from which at the most 20% of the maternal cells thereof have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells.

Thus, according to the invention a method is obtained whereby the risk of removing fetal cells from the maternal blood sample before analysing the sample has been greatly reduced.

Furthermore, the present invention relates to a method of culturing fetal cells. The method comprises multiplying fetal blood cells in a cell culture comprising fetal blood cells and other cells, in particular maternal blood cells, the method comprising performing the culturing in a culture medium comprising hepatocyte growth factor or an equivalent thereof.

The multiplication method according to the invention may be carried out after isolation of the fetal cells by the method according to the invention or it may be used in connection with any other identification and isolation procedure. Also the multiplication method may be used on samples, wherein some enrichment has been carried out before and/or after the identification of the fetal cells.

Detailed description of the invention

Th present invention reveals a novel method of isolating fetal cells from maternal blood. Th present method has proven to be optimised in relation to isolating meth-

ods described in the prior art, and the present invention represents a method having beneficial properties technically and financially. In the light of th naturally occurring ratio between maternal cells and fetal cells the present invention presents a method wherein fetal cells are isolated from maternal blood without prior enrichment or concentration of the sample, providing for a method of isolation by which the risk of losing fetal cells due to enrichment or concentration procedure has been reduced.

A major difference between maternal and fetal red blood cells is the latter having a nucleus, i.e. maternal red blood cells are anucleated. Furthermore, maternal blood contains three types of nucleated fetal cells, nucleated erythrocytes, syncytiotrophoblasts and lymphocytes. It is an object of the present invention to provide for a method wherein the blood sample from which the fetal cells are isolated is substantially not enriched or concentrated prior to isolation by removing any of the maternal cells.

Accordingly, in order to reduce the risk of removing fetal cells in the preparation of maternal blood samples to be analysed it is an object of the present invention that at most 20 % of the maternal cells of the maternal blood sample have been removed or will be removed before or after the labelling of the fetal cells, thus that substantially no enrichment of the sample is carried out before identification of the cells. In a more preferred embodiment at most 15 % of the maternal cells have been removed, such as at most 10 % of the maternal cells, more preferred at most 5 % of the maternal cells, more preferably at most 2.5 % of the maternal cells, most preferred at most 1 % of the maternal cells.

According to the invention it is even more preferred that substantially none of the maternal cells have been removed from the sample.

Thus, it is encompassed by the present invention that at most 20 % of the maternal nucleated blood cells have been or will be removed and/or at most 20 % of the anucleated red blood cells have been or will be removed.

Even more preferred is a method wherein substantially none of the nucleated blood cells or anucleat d r d blood cells have been removed from the sample. Thereby th sample may be used as such directly after taking the maternal blood sample.

It is desirable to obtain as large a maternal blood sample as possible in order to increase the total number of fetal cells. However, due to proctical problems the sample must be with certain limits. Accordingly, the size of the maternal blood sample is preferably in the range of 5 to 40 ml, such as from 10 to 30 ml.

Also, according to the invention it is preferred to dilute the sample before labelling or before identification of the fetal cells (to facilitate the identification of the fetal cells). The sample may be diluted at least 1.5 times, such as twice, more preferred at least three times, such as five times by adding isotonic buffers, such as saline solutions, phosphat buffered saline solutions, PBS, and/or suitable growth media, such as basal media, and tissues growth media.

The selective labelling of the fetal cells may be carried out by any suitable method. Fetal cells may be distinguished from maternal cells by the specific recognition of a fetal cell antigen or they may be distinguished by staining with a labelled antibody to a protein selectively produced by fetal cells or they may be distinguished from maternal cells by the specific recognition of DNA or RNA encoding a protein selectively produced by fetal cells.

Accordingly, it is an object of the present invention to provide for the selective labelling of fetal blood cells in the maternal blood sample based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

Preferably, fetal-cell-specific RNA sequences are used as fetal cell markers. Such RNA is generally messenger RNA (mRNA). The presence of such RNA indicates that the gene for the fetal protein is being transcribed and expressed. (The probes used to identify fetal cells in a sample containing fetal and maternal cells include nucleic acid molecules, which comprise the nucleotide sequence complementary to the nucleotide sequence of the RNA molecule encoding a specific protein. Fetal cells contain distinct mRNAs or RNA species that do not occur in other cell types. The detection of these RNAs, whether as mRNA can serve to identify cells, or even subcellular fractions of cells fetal or embryonic in origin). According to the present invention th m-RNA is coding for a protein selected from the group consisting of

embryonic hemoglobin, such as ε and zeta hemoglobin nd fetal hemoglobin, such as gamma and alpha hemoglobin.

Further, according to the present invention DNA probes (oligo) for the hybridisation are directed against embryonic cell RNA, such as for ε and zeta hemoglobin and for fetal hemoglobin, such as for gamma and alpha hemoglobin. A DNA probe is synthesised as an oligodeoxynucleotide using a commercial synthesiser. Probes may be comprised of the natural nucleotide bases or known analogues of the natural nucleotide bases.

Yet further to the invention the hybridisation probe is selected from peptide nucleic acid (PNA) probes and other synthetic molecules capable of Watson Crick-base pairing with the fetal m-RNA.

In one embodiment of the invention a synthetic DNA probe is employed to which chromofluors have been covalently attached. The binding of such probes to the cell may be observed under a microscope as a bright fluorescence or may be detected by a fluorimetric apparatus.

In another embodiment the DNA probes are directly labelled, or they may be indirectly labelled with enzymes, such as alkaline phosphatase. Such probes may bind to fetal RNA.

Certain RNA populations are present in high abundance and other fetal or embryonic-specific RNAs are present in low abundance. Several RNA species occur simultanously in fetal cells as opposed to maternal cells. This provides for yet another method of enhancing the distinction between fetal cells and non-fetal cells by the detection of multiple RNA species. Two or more RNA species may be detected using one or more probes for a first RNA sequence and one or more probes for a second RNA sequence. The probes for the first sequence are labelled to provide a first signal, such as a greenish fluorescence, and the probes for the second sequence are labelled to provide a signal that is different from the first signal, such as a reddish fluorescence. When the combination of both signals are detected in a single cell, which in this case would be an orange fluorescence, then both RNAs are found and thus a fetal cell has been detected.

According to another method of the inv ntion the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells. Such a protein may be selected from the group consisting of embryonic hemoglobin, such as ε and zeta hemoglobin and fetal hemoglobin, such as gamma and alpha hemoglobin.

In particular the labelling may be carried out by the use of an antibody selected from antibodies against various types of normal globin chains in human hemoglobin, for example anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, and monoclonal FITC labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal FITC labeled antibodies, and TRITC labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal FITC labeled antibodies, and TRITC labeled antibodies, anti alpha (α) antibodies, and anti beta (β) antibodies.

In a preferred embodiment of the present invention the labelling is carried out using anti epsilon (ε) monoclonal antibodies or anti zeta (ζ) monoclonal antibodies, more preferably anti epsilon (ε) unlabelled monoclonal antibodies or anti zeta (ζ) unlebelled monoclonal antibodies, or anti epsilon (ε) monoclonal biotin labelled antibodies or anti zeta (ζ) monoclonal biotin labelled antibodies.

In another preferred embodiment of the invention the labelling is performed using anti gamma (γ) FITC labeled antibodies.

In order to enhance the probability and selectivity of identifying the fetal cells or the background of maternal cells by the labelling two or more selective labellings may be performed. The combination of two or more labellings may be a combination of any of the labellings used for single labelling as well. Accordingly, the combined labelling may be carried out by the use of two or more different hybridisation probes, such as a combination of a DNA probe and a PNA probe for hybridisation with the same fetal RNA or more preferred with different RNAs. Also, two or more different DNA probes (or PNA probes) may be used for hybridisation with different fetal RNAs.

The enhanced selective labelling may also by carried out by the use of two or more antibodies directed against the same protein or different proteins. In this embodiment the labelling with two or more labels may be carried out simultaneously.

In another embodiment a combination of an immunological labelling and a hybridisation labelling may be employed according to the present invention. In this embodiment the labelling is normally carried out sequentially by a first immunological labelling step, then identification of the labelled cells, and then a second step of hybridisation labelling for verification of the identification of the cells labelled by the first step. It is preferred in the first step to use antibodies against epsilon, gamma and/or zeta globin and in the second step to use hybridisation for epsilon and/or zeta globin to verify the fetal cells identified.

An important feature of the present invention is the identification of the labelled fetal cells without the sample being enriched or concentrated with respect to the fetal cells in order to avoid loss of fetal cells. Accordingly, the method according to the present invention comprises identification of the selectively labelled fetal cells.

In one embodiment the identification is performed by spreading the blood sample on a solid surface and detecting the labelled cells on the surface. The detection may be carried out by any suitable means in accordance with the labelling method in question. The choice of solid support surface may depend upon the procedure for visualisation of the cells. Some materials are not uniform and therefore shrinking and swelling during in situ hybridisation procedures will not be uniform. Other autofluoresce support materials will interfere with the determination of low level fluorescence. Support materials according to the invention may comprise glass, nylon, nitrocellulose and Scotch tape, and any suitable membranes, such as filtermembranes. Preferably, the collected samples are spread on a support surface in a monolayer for the cells not to overlap one another.

Various antibodies have been used to discriminate between maternal and fetal cells. The antibodies may be coupled to numerous solid surfaces or supports/substrates, such as containers, columns, wells, beads, or particles by physical or chemical bonding. Alternatively, the antibodies may be coupled to a material, which facilitat s

the separation step. For example antibodies may be labelled with fluorescent markers and cells to which these labelled antibodies bind may thereby be separated with a cell sorter according to known procedures.

The in situ hybridization process according to the invention may be where a fetal nucleic acid is made available by having the fetal cell attached to a solid support. The material can be detected directly by for instance in situ hybridisation. The nucleic acid of the fetal cells can be amplified prior to detection using a known amplification technique, such as the polymerase chain reaction (PCR). Primers for PCR amplification are chosen to specifically amplify a DNA of interest in the fetal DNA.

Due to the large amount of cells to be examined to find the small amount of fetal cells in the blood sample an important factor for the detection equipment is the rate of cells identified per unit of time. For example very fast scanning microscopes may be used for the identification.

Preferably, during or after identification of the fetal cells the position of detected labelled cells on the surface is recorded. This provides for the later collection of the detected cells from the position which has been recorded. Thereby it is possible to identify and specifically isolate substantially only the selectively labelled fetal cells.

The position of the detected labelled cells on the supporting surface may be recorded by use of a scanner provided with photo-multipliers registering light. It is of importance for the use of the method that a fast scanning system is used, for example a scanner capable of scanning appr. 1m/sec.

According to another embodiment the identification of the selectively labelled cells is performed on a suspension of the cells in a liquid, in liquid portions of which one or more fetal cells are identified. The labelled cells may then be separated from the maternal cells branching off at least one subflow from a flow of the blood sample, said subflow containing at least one labelled fetal cell. Several of said subflows may then be combined to obtain a volume containing several fetal cells.

The cells collected according to any of the procedures may be subjected to further identification and/or investigation, such as microscopic and/or molecular identifica-

tion and/or investigation. Th cells may be subjected to investigations of analysing the presence of genetic diseases, for example. The nucleic acid of fetal cells may be analysed for diagnostic or other purposes. For instance the presence or absence of a particular nucleic acid may indicate the presence of certain genes coding for diseases, such as cystic fibrosis. The nucleic acid may additionally be analysed for X or Y specificity. Thus, the presence of a Y chromosome encoded genes or gene products is a qualitative distinguishing feature of the cells of a male fetus.

Verification of the selective identification of fetal cells may be carried out by several methods. In a model system the method may be performed on maternal blood samples from pregnant women carrying a male fetus. The cells isolated may then be analysed for the presence of a Y chromosome, indicative of cells being from the male fetus.

Another verification method, which is usable independent of the sex of the fetus, is verification by use of identification of small tandem repeats (STR) or variable number tandem repeats (VNTR) to detect genetic input from the father, thereby verifying fetal cells, as the only cells in the sample comprising input from the father.

As may be understood from the above the present method may be carried out for the isolation of any kind of rare event cells in a blood sample, and is particular interesting when used for rare event cells being present in very low concentrations, such as those for the fetal cells in maternal blood. This may for example be true for some cancer forms.

Another object of the present invention is providing a method for multiplying fetal blood cells, preferably fetal red blood cells, in a cell culture comprising fetal blood cells and other cells, in particular maternal blood cells. The cultivation may be carried out on any sample comprising fetal blood cells, such as umbilical cord samples, maternal blood samples.

The multiplication of the fetal cells is desirable to achieve due to the use of the present invention. The method according to the invention comprises performing the cell culturing in a culture medium containing h patocyte growth factor or an equivalent thereof.

The culture medium may be any suitable cell culture medium, such as RPMI 1640, Iscove's MDM with or without methyl cellulose, alfa DMEM, or any other medium comprising basal vitamins and minerals. pH of the growth medium is appropriately about physiological pH of 7.4.

The preferred temperature of culturing is about 37 °C and preferably the incubator has a CO_2 level of 5 % and a high humidity. The culturing period is preferably between 3-20 days, such as from 4 to 8 days. The medium may be used during the whole culture period without being changed.

The present inventors have found that the presence of hepatocyte growth factor or an equivalent thereof enhances the growth of fetal cells. By the term "equivalent growth factor" is meant a growth factor or growth factor-like substance, which, when culturing cells of a blood sample from a pregnant woman carrying a male fetus, will give rise to an increase in the concentration of fetal cells as compared to cultivation of a blood sample without the growth factor or growth factor-like substances.

The hepatocyte growth factor may be added to the medium at the beginning of the growth of the cells. However, hepatocyte growth factor may also be added later during the growth of the cells immediately before the development stage of the cells demanding the factor.

Furthermore, in order to grow fetal red blood cells the medium must contain erythropoietin, which is of importance for the development of fetal red blood cells. Also CSF is of importance in the medium.

The concentration of hepatocyte growth factor or an equivalent thereof in the culture medium is preferably between 0.1 and 2000 ng per ml medium, more preferably 5 - 1000 ng per ml medium, such as more preferably approx. 20 ng per ml medium.

The culture method according to the invention is preferably a method for selectively culturing fetal cells, i.e. that the multiplication of fetal cells is more rapid than the multiplication of maternal cells, or even that the growth of maternal cells is supressed as compared to that of the fetal cells.

The method of culturing fetal cells may be combined with the method of selectively isolating fetal cells in any suitable manner. Accordingly, a maternal blood sample may be subjected to cultivation of fetal cells before subjecting the sample to labelling, identification and isolation of the fetal cells. Thereby the starting concentrations of fetal cells in the maternal blood sample are increased without removing any cells originating from the mother. The culture method may also be used in connection with analysis of maternal blood samples that have been pretreated in order to remove some of the maternal cells.

Also, the culture method may be conducted on fetal cells having been identified and isolated according to the isolation method described above, in order to increase the amount of fetal cells for further analysis.

After cultivation the fetal cells may be harvested by any suitable method. In case the colonies obtained are harvested collectively, there will be a very high number of cells of which only some will be from the fetus, and therefore some kind of enrichment procedure may be necessary. Contrary to this, colonies may be harvested individually to increase the rate of fetal cells in the harvested material.

The invention is further exemplified by the following non-limiting examples.

Examples

Example 1

Cultivation of fetal cells

Fetal cells from maternal blood obtained after abortion due to detection of fetal disease or genetic abnormality.

Two pregnancies, one at 14 (case 1) and one at 17 (case 2) gestational weeks, were studied. In case 1, an ultrasound analysis showed anencephalia in a male fetus. The sex was confirmed by fetal karyotyp . In case 2, cytogenetic analysis of th

amniotic fluid showed Klinefelter Syndrome: The amniocentesis was performed 25 days before th blood samples were shown.

Procedure

Blood samples: 10-15 ml of peripheral venous blood was obtained before and after therapeutic abortion. The blood was collected into sterile heparinized vacutainer tubes. In case 1, the blood samples taken before and after abortion were mixed, whereas in case 2 the two samples were processed separately. The blood samples were diluted 1:1 in phosphatebuffered saline (PBS)and overlayered onto the gradient material. Mononuclear cells were isolated from the blood by density gradient centrifugation at 550g for 30 minutes at room temperature using Histopaque 1.107 g/ml (Sigma, St. Louis, USA). After centrifugation the cell-containing layers were collected; the cell suspension was washed twice in PBS and centrifuged at 550 g for 10 minutes.

Cell culture: mononuclear cells were plated in 35 mm Petri dishes with 3 ml of Iscove's DMEM containing 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 0,9 % methylcellulose, 2 nM L-glutamine, 50 ng/ml human recombinant stem cell factor, 20 ng/ml human recombinant GM-CSF, 20 ng/ml human recombinant IL-3, 20 ng/ml human recombinant IL-6, 20 ng/ml human recombinant G-CSF, 3 units/ml human recombinant erythropoietin (StemCell Technologies Inc. Vancouver, Canada). Cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. Colonies were identified and counted under an inverted microscope at culture 4 and 5. In case 1, CFU-E derived colonies (~100 per dish) were detected and harvested at culture day 5. In case 2, BFU-E (~80 per dish) and CFU-GM (~20 per dish) –derived colonies were observed and harvested at culture day 4 and 5. The harvested cells were then sorted as described.

Collective colonies harvesting: 1-2 ml of PBS were dispersed in the dishes. Cells and media were removed and further 5 ml of PBS were added and the cells resuspended. Cells were then washed twice with PBS.

CD71 selection: CD71 is an antibody expr ssed by fetal cells. By using a anti-CD71 antibody it is possible to detect the fetal cells in the cultures. Cell fraction may be

CD71 positive or CD71 negative. The selection of CD71 was carried out according to the instructions of Miltenyi Biotec GmbH, Bergisch Gladbach, G rmany.

Slide preparation and Fluorescence in situ hybridization (FISH): the number of cells in the CD71 positive and negative fractions was estimated using a Bürker counting chamber. Approximately 200.000 cells were resuspended in 500ml of PBS, pipetted into the centrifugation bucket (Eppendorf, Hamburg, Germany) and cytocentrifuged at 40g for 2 minutes. After airdrying, the slides were fixed in methanol for 10 minutes at -20°C, post-fixed in 2% phosphate buffered paraformaldehyde for 10 minutes at R.T. and dehydrated in ethanol.

Two-colour FISH was performed using a Spectrum Orange labelled alpha-satellite probe (DXZ1) for centromere region Xp11.1-q11.1 and a Spectrum Green labelled satellite III probe for the Yq12 region according to the manufacturer's instructions (Vysis, Downers Grove, IL, USA). Slides were mounted in Vectashield (Vector laboratories, Burlingame, CA, USA) containing DAPI and analyzed under a Leica fluorescence microscope (x 40) using a double-bandpass filter.

Fetal cell count: the hybridized slides from the CD71 positive and negative fractions were analyzed. The whole cytospin area was analyzed and the total number of fetal cells counted. Fetal cells were identified by the presence of the Y signal and classified as nucleated red blood cells, granuloctyes, monocytes-macrophages according to their morphology.

Results

CD71 + fraction

Case 1:

The total number of cells in the CD71 positive fraction was $6x10^5$. Approximately $4x10^5$ cells were analyzed and 49 fetal cells (Y positive by FISH) (Table 1) were detected. 48 of these fetal cells were classified as nucleated red blood cells according to their morphology, whereas one was a fetal macrophage.

Case 2:

In the pre-abortion sample the total number of cells in the CD71 positive fraction were $1x10^5$. Approximat ly $5x10^4$ cells were analyzed in the CD71 positive fraction. No fetal cells could be detected in any fraction.

In the post-abortion sample the total number of cells in the CD71 positive fraction was $1x10^5$: The cell number analyzed was approximately $5,6x10^3$ and 5 fetal cells were detected and classified as nRBCs (Y positive by FISH) (Table I).

CD71 – fraction

Case 1:
The total number of cells in the negative CD71 fraction was $4.5x10^6$ and the cell number analyzed was approximately $4x10^5$. Only one fetal cell, classified as a monocyte, was detected.

In the pre-abortion sample the total number of cells in the CD71 negative fraction was $3x10^6$. Approximately 40x104 cells were analyzed in the CD71 negative fraction. No fetal cells could be detected in any fraction.

In the post-abortion sample the total number of cells in the CD71 negative fraction was $5x10^5$ and out of $5x10^4$ cells of the total number that were analyzed, 9 fetal cells were identified by FISH; 5 of these were classified as macrophages, 2 as granulocytes and 1 as nRBC.

Table 1: Number of fetal cell obtained after culture of mononuclear cells isolated from two maternal blood samples.

Mononuclear cells were isolated by density gradient, cultured in methylcellulose and subsequently selected by magnetic cell sorting to give a CD71+ and CD71- fractions as described in Materials and Methods. Cytospin slides were obtained and FISH analysis performed. The number of fetal cells in the CD71+ and CD71- fractions was counted.

CD71+ fractions:

Case	Sample (pre-post) abortion	Number of cells in the CD71+ fraction	Number of cells analysed	Number of fetal (male) cells in the CD71+ fraction
1	pre + post	6×10^5	400.000	49
2	pre	1×10^6	50.000	0
	post	1×10^5	5600	5

CD71- fractions:

Case	Sample (pre-post) abortion	Number of cells in the CD71- fraction	Number of cells analysed	Number of fetal (male) cells in the CD71- fraction
1	pre + post	4.5×10^6	400.000	1
2	pre	3×10^6	50.000	0
	post	5×10^5	50.000	9

Conclusion

Considering the very low number of fetal cells in maternal blood as discussed above the relatively higher numbers found after culturing the samples indicates the significance of the cultivation method. The increase in the observed number of fetal cells is believed to be due to the semisolid medium and the addition of fetal bovine serum and various cytokines including erthropoietin and hepatocyte growth factor (HGF)[4].

Example 2

Detection of fetal cells for Y chromosomes

Fetal cells were diagnosed by Y chromosome FISH analysis followed by the classification of the cell morphology.

In the first case the pre- and post-abortion samples were mixed to increase the possibility of finding Y positive cells after culturing. A There Y chromosome was present in 50 cells. In the second case there was no Y chromosome present in the preabortion sample after culturing. However, in the postabortion sample there was a Y chromosome present in 14 cells.

Claims:

1. A method for isolating fetal cells from maternal blood, comprising performing, on a sample of maternal blood from which at the most 20 % of the maternal cells thereof have been removed, selective labelling of fetal cells in the maternal blood sample, identifying the selectively labelled fetal cells, and specifically isolating substantially only the selectively labelled fetal cells.

2. The method according to claim 1, wherein at the most 15 % of the maternal cells thereof have been removed.

3. The method according to claim 1, wherein at the most 10 % of the maternal cells thereof have been removed.

4. The method according to claim 1, wherein at the most 5 % of the maternal cells thereof have been removed.

5. The method according to claim 1, wherein at the most 2.5 % of the maternal cells thereof have been removed.

6. The method according to claim 1, wherein at the most 1% of the maternal cells thereof have been removed.

7. The method according to claim 1, wherein substantially none of the maternal cells have been removed from the sample.

8. The method according to any of the preceding claims, wherein at the most 20 % of the maternal nucleated blood cells thereof have been removed.

9. The method according to any of the preceding claims, wherein at the most 20 % of the anucleated red blood cells thereof have been removed.

10. The method according to the claims 1-9, wherein substantially none of the anucleated blood cells have been removed from the sample.

11. The method according to any of the preceding claims, wherein at the most 20% of the anucleated red blood cells have been removed from the sample, and at the most 20% of the nucl ated blood cells have been removed from the sample.

12. The method according to any of the preceding claims, wherein the maternal blood sample is diluted before labelling or identification of the fetal cells.

13. The method according to any of the preceding claims, wherein the selective labelling is based on hybridisation of a probe to m-RNA selectively expressed by fetal cells.

14. The method according to claim 13, wherein the m-RNA is m-RNA coding for a protein selected from the group consisting of embroynic hemoglobin such as ε and zeta hemoglobin and fetal hemoglobin such as gamma and alpha hemoglobin.

15. The method according to claim 13 or 14, wherein the hybridisation probe is selected from DNA probes, PNA probes, and other synthetic molecules capable of Watson Crick-base pairing with the fetal m-RNA.

16. The method according to any of claims 1-12, wherein the selective labelling is based on an antigen-antibody reaction with a protein selectively produced by fetal cells.

17. The method according to claim 18, wherein the protein is a protein selected from the group consisting of embroynic hemoglobin such as ε and zeta hemoglobin and fetal hemoglobin such as gamma and alpha hemoglobin.

18. The method according to claim 16 or 17, wherein the antibody is selected from anti epsilon (ε) antibodies, such as monoclonal unlabelled antibodies, and monoclonal FITC labeled antibodies, anti zeta (ζ) antibodies, such as monoclonal unlabelled antibodies, monoclonal biotin labelled antibodies, monoclonal FITC labeled antibodies, and TRITC labeled antibodies, anti gamma (γ) antibodies, such as polyclonal (sheep) antibodi s, such as monocl nal unlabelled antibodi s, monoclonal biotin labell d antibodies, monoclonal FITC labeled antibodies, and TRITC labeled antibodies, anti alpha (α) antibodies, and anti beta (β) antibodies.

19. The method according to any of the preceding claims, wh rein two r more selective labellings are performed to enhance the probability of identifying the fetal cells in the sample.

20. The method according to claim 19, wherein a labelling with a hybridisation probe is combined with a antigen-antibody labelling.

21. The method according to any of the preceding claims, wherein the identification of the selectively labelled fetal cells is performed by spreading the blood sample on a solid surface and detecting labelled cells on the surface.

22. The method according to claim 21 wherein the position of detected labelled cells on the surface is recorded.

23. The method according to claim 22, wherein the detected cells the position of which has been recorded are collected.

24. The method according to any of claims 1-20, wherein the identification of the selectively labelled cells is performed on a suspension of the cells in a liquid, in liquid portions of which one or more fetal cells are identified are collected.

25. The method according to claim 24, wherein the labelled cells are separated from the maternal cells by establishing a flow of the blood sample and branching off a subflow containing at least one labelled fetal cell.

26. The method according to claim 23 or 24, wherein at least some of the cells thus collected are subjected to microscopic and/or molecular identification and/or investigation.

27. A method for multiplying fetal blood cells in a cell culture comprising fetal blood cells and other cells, in particular maternal blood cells, the method comprising performing the culturing in a culture medium comprising hepatocyte growth factor or an equivalent th reof.

28. The method according to claim 27, wherein hepatocyte growth factor is added to the medium after at least two days of growth.

29. The method according to claim 27 or 28, wherein fetal red blood cells are multiplied.

30. The method according to claim 27 or 28, wherein the medium further comprises erythropoietin.

31. The method according to any of the claims 27-30, wherein the medium further comprises CSF.

32. The method according to any of the claims 27-31, wherein the concentration of the hepatocyte growth factor is between 0.1 and 2000 ng per liter.

33. The method according to any of claims 27-33, wherein the fetal cells have been isolated by a method as defined in any of the claims 1-26.